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(54) METHOD FOR DETECTING AND SEPARATING ANTIGENS AND ANTIBODIES IN BLOOD OR OTHER SAMPLES

(71) We, COULTER ELECTRONICS INC., a corporation organised and existing under the laws of the State of Illinois, United States of America, of 590 West 20th Street, Hialeah, Florida, United States of America, do hereby declare the invention, for which we pray that a patent may be granted to us, and the method by which it is to be performed to be particularly described in and by the following statement:—

This invention relates to methods for testing samples to determine the presence of disease-related antigens and antibodies and more particularly to methods for simultaneously identifying the various disease-related antigens and antibodies present in a sample.

Many diseases which occur are caused by or accompanied by the entrance of antigen organisms into the body and into the blood stream. An antigen is a protein or carbohydrate substance which may be part of an organism such as a parasite, bacterium or virus particle, or a drug or other nonbiological material which when introduced into the body, stimulates the production of an antibody specific to the antigen. An antibody is a globulin that combines specifically with and aids in the neutralization of the specific antigen. A "globulin" is a protein insoluble in water but soluble in salt solutions.

In order to detect a number of diseases, samples of a body fluid such as blood or spinal fluid or samples of a body tissue are taken from the patient and tested to determine the presence of certain antigens or antibodies known to be present only during or after the occurrence of a specific disease. If the antigens or antibodies are detected, current or past presence of the disease is verified. Furthermore, a quantitative estimate of the antibody or antigen can be obtained. If the amount is quantified over a period of days, it can be seen whether the antibody or antigen production is constant indicating a prior infection or static condition; or is increasing indicating the degree of infection is increasing and any prescribed treatment is ineffective; or is decreasing indicating the degree of infection is decreasing and any prescribed treatment is effective.

A number of methods for determining the presence of antigens or antibodies in samples such as blood have been developed. One such method is described in a U.S. Patent No. 3,088,875 issued to Roy T. Fisk, May 7, 1963. In this patent, small plastic particles, called microspheres, are coated with an antigen related to a specific disease. The sample under test then is added to the microspheres. If the sample contains antibodies related to the specific disease, the antibodies will be attracted to and attach to the antigen coated microspheres. This attachment will cause the microspheres to begin to attach to one another thus giving rise to a visible aggregation of particles. If the particles aggregate in this fashion, presence of the specific disease is confirmed.

Although this technique has been used effectively, it is a subjective test that requires human observation as to when particle aggregation has taken place. A weak reaction might not be recognized by this technique. The test must be separately performed for each disease of interest so that a great deal of time must be spent in a full analysis, and the test method is not readily adapted to an automated process which will reduce costs, time expended and eliminate subjective determinations.

The present invention provides a method of testing a sample of a biological substance obtained from an animal to determine the presence or absence of a disease or diseases therein, which comprises

(a) coating the members of each group of a number of distinguishable groups of particles with an antigen or an antibody corresponding to a particular disease, the members of each group being coated with a different antigen or antibody.

(b) incorporating the sample to be tested with a mixture of the groups of coated particles whereby any antibodies and antigens

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present in the sample attach themselves to the members of that group of particles coated with the corresponding antigen or antibody, respectively,

(c) incorporating the product resulting from (b) with a labeled substance which has an affinity for an attached antibody or antigen, and

(d) passing the particles through a sensing device which is capable of sensing the presence or absence of the label and which is also capable of identifying any particular group of particles to which the labeling substance may have become attached, whereby the presence or absence of a particular disease is detected.

The particles to be coated are especially plastics and, in particular, microspheres.

It will be appreciated that there may be used any detectable label. Substances used for labeling antibodies and antigens are well known in the art.

U.S. Patent No. 3,790,492, issued to M. J. Fulwyler, on February 5, 1974, describes a method for the production of uniform diameter microspheres. The patent further describes a method for producing uniform microspheres having selected diameters in the range of 2 to 40 micrometers. Uniformly shaped microspheres of such small diameter had not been previously producible. These spheres are extremely important in the method of the invention as will be explained subsequently in greater detail.

In one embodiment of the method of this invention, a number of groups of microspheres are employed, with each group containing microspheres of a particular size range, for example, three groups of uniformly sized microspheres may be employed, the first group consisting of approximately 10,000 microspheres having a diameter of 4—5 micrometers, the second group consisting of 10,000 microspheres having a diameter in the range 7—8 micrometers; and the third group consisting of 10,000 microspheres having a diameter of 10—11 micrometers.

Each group of microspheres is coated with a different specific disease-related antigen or antibody by any of a number of methods such as, for example, the method taught by R. T. Fisk, noted previously. The generally used coating method comprises suspending the microspheres in an alkaline buffer solution with a test antigen. The suspension is stored, and, after a suitable period, washed to remove excess solution and leave only the coated microspheres.

A sample, for example, of blood or blood serum, which is to be tested for the presence of the three diseases related to the antigens coated onto the microspheres then is incorporated with a mixture of all three groups of microspheres. Generally a single drop of test material is added to the mixture of microspheres. If any of the three diseases is or has been present, antibodies will be found in the sample as described previously. These antibodies will become attached to the corresponding antigen coated on the microspheres. For example, a group of microspheres of a particular size, called group A, is coated with antigen A related to disease A; if disease A is or was present, A antibodies will be present in the blood and will become attached to the group A microspheres. Similarly, a further group of particles, having a different size and called group B, coated with antigen B related to disease B will attach antibodies B, if such antibodies are present in the blood. The same is true for a further group of differently sized particles, group C. After a time has passed sufficient to allow any antibodies in the sample to attach themselves to the microspheres, the microspheres may be washed to remove any excess sample.

Next, any attached antibodies are labeled. This is preferably done by contacting the treated microspheres with labeled antibodies directed against the attached, generally human, antibodies. Rabbit antibodies directed against human antibodies are preferred. A suspension of "rabbit anti-human antibodies" may be prepared in a manner such as taught by R. T. Fisk (loc. cit.). Again, the actual conditions, for example, pH and buffer, are dependent on the actual system tested. The labeling antibody preferably rabbit anti-human antibody has preferably been treated with a fluorescent label, for example, fluorescein isothiocyanate, for example, as taught by M. Goldman in "Fluorescent Antibody Methods", Academic Press, 1968 so that it will fluoresce in the presence of particular wavelengths of light. Rabbit anti-human antibody has an affinity for all huamn antibodies and will attach to those microspheres which have absorbed any human antibody from the sample.

Multiparameter particle analysis machines are described by Steinkamp, *et al* in *Review of Scientific Instruments*, Volume 44, No. 9, September, 1973, pages 1301 to 1310. In these devices, particles such as the above described microspheres, suspended in a liquid, for example, a saline solution flow one at a time past several electro-optical sensors. Certain of the devices allow the particles to pass through a particle detector for example, as shown and described in U.S. Patent No. 3,259,842. In this type of particle detector, particles pass through a microscopic aperture generally having a diameter of approximately 100 micrometers. Each particle passing through the aperture produces a signal in the form of a pulse with amplitude related to the particle size or

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volume. Particles in this apparatus are subsequently passed through a laser beam causing any fluorescent dyes attached to the particle to emit fluorescent light. Photodetectors sense emitted fluorescent light and develop signals which are coupled to control circuitry which correlates, for each particle, size or volume as represented by the associated pulse amplitude, and presence or absence of fluorescence in order to identify each particle.

In the method of this invention, after suitable incubation time for the attachment of the labeled anti-human antibody, the microspheres are placed in suspension in, for example, a saline solution and passed through an appropriate sensing device. In the case of a fluorescent label, a device as described above may be used. The device will detect the size and volume of each microsphere as it passes through a particle detector of the type described above. If the particle has absorbed fluorescent anti-human antibody, it will fluoresce when passed through the laser beam. The fluorescence will be detected and coupled to the control circuitry where the fluorescent signal will be correlated with the size detemined for that particular microsphere. The presence of a fluorescent signal will trigger the control apparatus to indicate the presence of the particular disease related to the antigens and antibodies attached to the microspheres in that particular size group. It should be understood, of course, that occasional inconsistencies may be recorded so that the machine may be adapted to register the presence of a particular disease antigen or antibody only when a predetermined number of microspheres in a specific size range have fluoresced or passed through the laser beam and the fluorescence has been detected and correlated to the particular particle size. Of course, if more than one particle size fluoresces when passed through the laser beam, the detections of antibodies or antigens associated with the particle sizes in these groups will also be indicated by the control apparatus.

The above described multiparameter analysis apparatus additionally may be combined with a particle separator apparatus such as is shown and described in U.S. Patent No. 3,380,584. When combined with a particle separator apparatus such as described in the aforementioned patent, the microspheres may be separated and collected in accordance with each size group for allowing separate study and analysis of the attached antibodies.

Alternatively, according to the method of the invention, an antibody associated with a specific disease may be coated onto the microspheres of a particular size group rather than an antigen. The sample to be tested then can be added to the groups of microspheres, and the subsequent procedure carried out analogously to that using antigen-coated microspheres, for example, as follows: If the sample contains antigens related to the specific diseases being tested for, the antigens in the sample will be attracted to and attached to the antibodies coated onto the various microspheres. After a time has passed sufficient to allow the sample carried antigens to be attracted to and attached to the antibody coated microspheres, the mixture of microspheres may be washed to remove excess sample. A solution of an appropriate, labeled reagent, for example, a reagent treated to fluoresce, is then added to the microspheres. The reagent has affinity for one or more of the sample antigens attached to the microspheres so that the reagent will attach to these microspheres. After a suitable incubation time the microspheres are passed through the multiparameter particle analysis machine. If microspheres in a particular size group indicate the presence of a label, for example, if they fluoresce, this will be detected by the apparatus thus indicating the presence of that particular disease aiding the physician in determining the proper treatment.

It should be understood that although the above described method requires distinctions of microsphere size and presence of a label, for example, a fluorescent label, it may be equally desirable to provide identification for particle size and absence of label, thus identifying absence of a disease. In another variation of the method of the invention, the size can be the same and each particle group can be separately labeled with separate photodetectors provided in the analysis device to sense the microsphere labels, for example, colours may be used. In another alternative method, the microspheres can be magnetically coded and this coding may be detected in addition to the fluorescence.

This method may be used for the detection of diseases of various aetiologies, for example, rheumatoid arthritis, syphilis, and brucellosis.

Fisk (*loc. cit.*) describes coating particles with Cohn Fraction II to detect antibodies for rheumatoid arthritis. The microspheres may be similarly coated with the antigen of histoplasma capsulation for the detection of histoplasmosis, or the antigen of coccidioides for the detection of coccidiomycosis (San Josquin Valley Fever), the actual conditions, for example pH and buffer, being dependent on the system tested.

The method of testing which is described in this specification is applicable to the examination of whole blood intended, for example, for blood transfusion.

The following Example illustrates the invention:

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I. *Preparation of Reagents for Coating Microspheres*

(a) For the detection of Rheumatoid Arthritis

Laboratory tests for Rheumatoid Arthritis have been serologic tests for the detection of an antibody-like agglutinating factor commonly called the rheumatic factor (RF). Testing for the presence of RF has been based upon the fact that it reacts with Cohn Fraction II.

In preparing this reagent for covering the microspheres, the Cohn Fraction II is dissolved in saline (0.16M) containing 0.1 sodium azide as a preservative, to make a 10% solution. This solution is sterilized by filtration through a bacteria excluding agent.

(b) For the detection of Syphilis

Laboratory tests for Syphilis have included tests for antibodies of Treponema Pallidum. Treponema Pallidum is the spirochete responsible for Syphilis. Lyophilized Treponema Pallidum used in the serologic tests for the detection of Treponema Pallidum antibodies is commercially available. The lyophilized Treponema is dissolved in saline (0.16M) containing 0.1% sodium azide as a preservative to make a 10% solution.

(c) For the detection of Brucellosis.

Brucella organisms are responsible for intermittent fevers in man and are most commonly contracted by persons working with cows, pigs or goats. Laboratory tests for Brucellosis have been serologic tests for the detection of antibodies generated by infections with any one of the three species of Brucella organisms, Brucella abortus, Brucella suis or Brucella melitensis. The reagent used to test for Brucellosis is a formalin-killed or heat-killed suspension of Brucella abortus.

II. *Coating of the Microsphere*

Three separate size groups of microspheres are employed. The groups are 5, 8 and 11 micrometers in diameter respectively. These microspheres are taken from the stock solution and washed twice with glycine or borate-saline buffer at pH 8.2. Each group is treated separately and the final concentration of each group in buffer is adjusted to the order of 10^{10} spheres/10ml.

Each 10 ml. microsphere suspension is treated with an equal volume of one of the three coating reagents noted above. The suspension with coating reagent added is incubated for one hour (± ½hr.) at 41°C (± 16°C) with stirring during the incubation. The suspension is then centrifuged to separate the particles and the separated particles are again washed twice with the same stock buffer solution noted above to remove any excess coating reagent which has not adhered to the particles. In order to reduce any undesired background fluorescence which may occur during a later step in this method when the spheres pass through the particle analyser, a 10% w/v bovine serum albumin solution is added to the microspheres and this suspension is incubated as noted above. The addition of this solution acts to block the unreacted or uncoated sites on the microspheres, and further inhbits addition of serum proteins to the microspheres. After incubation, the concentration of the microsphere suspension is again adjusted to be of the order of 10^{10} spheres per ml.

III. *Addition of Test Suspect Serum.*

One drop (approximately 0.05cc) of serum to be tested is added to a 10cc aliquot of coated microspheres containing equal numbers of each group of coated microspheres. The aliquot is then vortexed for approximately 1 minute. After vortexing, the aliquot is spun to separate the microspheres. Upon separation, the microspheres are washed twice with a borate or glycine-saline buffer as noted above to remove any excess antibodies or antigens, in solution. The suspension is then adjusted to provide a concentration of particles of 10^{10} spheres/10ml.

IV. *Addition of Fluorescently Labeled Anti-Human Globulin*

One milliliter of rabbit anti-human globulin, treated to fluorescence as discussed previously, is added to the 10ml microsphere suspension. This combination is incubated for 10 minutes (± 5 mins.) at room temperature, with a working titre of approximately 1:400 being maintained. The suspension is then centrifuged to remove the labeled microspheres and the removed microspheres are twice washed with borate or glycine-saline buffer to remove any excess fluorescent anti-human globulin.

V. *Introduction Into Multi-Parameter Particle Analysis Machine*

The microsphere particle suspension obtained from step IV is adjusted to a concentration of 5×10^6 microspheres/5ml and is introduced into an analysis machine of the type described by Steinkemp. A positive response for fluorescence in particles of a particular size indicates that the subject from whom the sample was obtained has or has had the respective disease.

WHAT WE CLAIM IS:—

1. A method of testing a sample of a biological substance obtained from an animal to determine the presence or absence of a disease or diseases therein, which comprises

(a) coating the members of each group of

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a number of distinguishable groups of particles with an antigen or an antibody corresponding to a particular disease, the members of each group being coated with a different antigen or antibody.

(b) incorporating the sample to be tested with a mixture of the groups of coated particles whereby any antibodies and antigens present in the sample attach themselves to the members of that group of particles coated with the corresponding antigen or antibody, respectively,

(c) incorporating the product resulting from (b) with a labeled substance which has an affinity for an attached antibody or antigen, and

(d) passing the particles through a sensing device which is capable of sensing the presence or absence of the label and which is also capable of identifying any particular group of particles to which the labeling substance may have become attached, whereby the presence or absence of a particular disease is detected.

2. A method as claimed in claim 1, wherein the biological substance is whole blood.

3. A method as claimed in claim 1 or claim 2, wherein the biological substance is plasma, serum or spinal fluid.

4. A method as claimed in claim 1, wherein the biological substance is a tissue preparation.

5. A method as claimed in any one of claims 1 to 4, wherein the particles are plastics particles.

6. A method as claimed in any one of claims 1 to 5, wherein the groups of particles are distinguishable by size.

7. A method as claimed in any one of claims 1 to 6, wherein the labeled substance is a fluorescent substance having an affinity for at least one of the antibodies and antigens that may be present in the sample.

8. A method as claimed in claim 7, wherein the fluorescent substance has an affinity for an antigen.

9. A method as claimed in claim 7, wherein the fluorescent substance is a fluorescent antibody directed against human antibodies.

10. A method of testing a sample of a biological substance obtained from an animal to determine the presence or absence of a disease, carried out substantially as described herein.

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